Phytoalexin Biosynthesis Induced by the Fungal Glucan Polytran L in Soybean, Pea, and Sweet Pepper Tissues

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Methods for convenient mass elicitation of phytoalexin accumulation in tissues of soybean, pea, and sweet pepper were developed. Polytran L, a commercially produced glucan of fungal origin, was employed as the elicitor at 0.25% (w/v). Glyceollin was induced optimally in 5-day-old soybean seedlings, whereas pisatin and capsidiol were induced in split peas and fully developed, unripe fruits of sweet pepper, respectively.

Phytoalexins are low molecular weight antimicrobial compounds that are both synthesized in plants and accumulated after exposure to microorganisms (Paxton, 1981). Synthesis and accumulation of phytoalexins in plant tissues also can be induced by certain chemicals, e.g., heavy metal salts, and physical agents, e.g., UV irradiation and freezing, called abiotic elicitors (Bailey and Mansfield, 1982). Substances of microbial origin, including polysaccharides, proteins, and fatty acids (biotic elicitors), also induce phytoalexin synthesis and accumulation (Bailey and Mansfield, 1982; Paxton, 1988). Although a wide variety of phytoalexin elicitors have been chemically characterized, not all elicit a broad spectrum of species (Block et al., 1984).

Phytoalexins have been shown to inhibit growth of a variety of organisms such as fungi, bacteria, and nematodes. Phytoalexins also may be active against the microbial pathogens of animals including humans (Gordon et al., 1980). Phytoalexins and their derivatives also may have potential application as pesticides (Paxton, 1991). On the other hand, the toxicity and carcinogenic/mutagenic potentials of phytoalexins need to be evaluated before phytoalexins can be applied practically as protectants/chemotherapeutants (Bailey and Mansfield, 1982; Rosenkranz and Klopman, 1990).

The present studies were undertaken to develop procedures for mass elicitation of phytoalexins that would allow the production of these compounds in sufficient quantities for such biological testing. Polytran L (PL), a glucan produced by the fermentation of glucose by a selected species of the fungus *Sclerotium*, was used as the elicitor. This polymer is primarily a linear chain β 1–3 glucan, with 30–35% of the linear chain units bearing single appended glucose linked β 1–6. The M_r of the polymer is >500 000 (Jetco Chemicals, Corsicana, TX).

MATERIALS AND METHODS

Elicitation Procedures. *Soybean.* Soybean seedlings were treated with elicitor according to a procedure developed for elicitation of pea seedlings (Sweigard and VanEtten, 1987). Two hundred grams of seeds of soybean (*Glycine max* L. Merr. cv. Williams 82) was surface sterilized with 200 mL of 0.45% (w/v) sodium hypochlorite for 10 min, followed by three rinses with 200 mL of sterile water. Sterilized seeds were incubated in enamel trays lined with moist paper towels in 200 mL of water, and covered with aluminum foil, at room temperature in the laboratory. Five days later, the trays were flooded with 500 mL of a sterile 0.25% (w/v) Polytran L solution which, after 1 h, was removed by vacuum. The trays were again covered with aluminum foil and incubated for an additional 3 days at room temperature. Each treatment was replicated three times.

Pea. Green split peas (*Pisum sativum* L.), store purchased, were elicited by immersing 5 g of split peas in 10 mL of 0.25% (w/v) PL in a Petri dish and then incubated for 4 days at room temperature (three replicates for each treatment).

Sweet Pepper. Peppers (*Capsicum annum* L.) were elicited according to the procedure of Watson and Brooks (1984). Fully developed, unripe, healthy sweet pepper fruits, store purchased, were washed with deionized water and then rinsed with MeOH. After air drying, each pepper was injected with 30 mL of 0.25% (w/v) PL solution. Three peppers per treatment were incubated for 4 days at room temperature, with shaking on a culture shaker at 30 rpm.

Extraction and Assay of Phytoalexins. *Glyceollin.* Treated soybean seedlings were extracted in 3 times their weight of 5 mM K_2HPO_4 in 49% EtOH and then left overnight at room temperature. This extract was clarified by centrifugation, and aliquots were analyzed by HPLC (Spectra Physics) using an Ultrasphere octyl column (4.6 mm × 25 cm). Aqueous 58% (v/v) MeOH was used as eluant at a flow rate of 1 mL/min, and elutants were monitored at a wavelength of 280 nm. Glyceollin 1 was the predominant component and was quantified with reference to an external standard.

Pisatin. Pisatin was extracted by homogenizing elicited split peas with 10 times their weight of 95% EtOH and allowing the homogenate to stand overnight in the refrigerator at 4 °C. The homogenate was filtered through Whatman No. 1 filter paper, and the residue was extracted two more times with 50 mL of 95% EtOH. The extracts were pooled and dried in a rotary vacuum evaporator at 50 °C. Water (10 mL) was added to the residue and the aqueous phase was extracted three times with 20 mL of diethyl ether. The ether phases were pooled and dried in a rotary vacuum evaporator, and the residue was dissolved in 5 mL of EtOH. Pisatin was analyzed by HPLC using the column and conditions described above, except that eluants were monitored at 309 nm. Pisatin was quantified with reference to an external standard.

Capsidiol. Pepper fruits were cut into 3–4 cm pieces and extracted with EtOAc (2 mL/g of fresh weight of pepper) by shaking overnight at room temperature. The elicitor solution in the pepper, which contained capsidiol (Watson and Brooks, 1984) as assayed by TLC, was added to the extractant solution. EtOAc was decanted, and the pepper pieces were extracted two more times with EtOAc by soaking for about 8 h on a culture shaker at 30 rpm. All of the EtOAc fractions were pooled and evaporated on a rotary vacuum evaporator at 50 °C. The residue was dissolved in 5 mL of hexane/2-propanol (3:1 v/v). A 50-μL aliquot was spotted on a silica gel G TLC plate, and the plate was developed with an EtOAc solvent. The capsidiol spot was visualized by spraying one lane on the margin of the TLC plate with vanillin reagent (1 g of vanillin in 30 mL of MeOH plus 0.2 mL of concentrated sulfuric acid), followed by incubation in a 100 °C preheated oven for about 5 min (R_f value 0.35). The capsidiol spot developed a turquoise color. Appropriate capsidiol bands from the unsprayed region of the TLC plate were

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Table I. Phytoalexin Accumulation in Soybean Seedlings, Pea Cotyledons, or Pepper Fruit after Elucidation with 0.25% Polytran L[a]

elicitor	phytoalexin, μg/g of fresh wt		
	glyceollin (3 days)[b]	pisatin (4 days)	capsidiol (4 days)
water (control)	15.4 ± 9	57.9 ± 13	2.0 ± 1
Polytran L	158.5 ± 9	533.5 ± 71	94.0 ± 15

[a] Data represent means ± SD. [b] Optimal elicitation time established by Bhandal and Paxton (unpublished data).

Table II. Accumulation of Phytoalexins in Different Host Tissues after Treatment with Known Phytoalexin-Eliciting Agents

host tissue	elicitor	phytoalexin	yield, μg/g of fresh wt
soybean[a] (cotyledon)	*P. megasperma* (mycelial suspension)	glyceollin	457
soybean[b] (leaves)	*Pseudomonas glycinea*	glyceollin	384
pea[a] (pods)	$HgCl_2$ (10^{-4} M sol.)	pisatin	277
pepper[a] (fruit pieces)	*Monilina fructicola* (conidial suspension)	capsidiol	29
pepper[c] (whole fruit)	cellulase	capsidiol	77

[a] Data from Bloch et al. (1984). [b] Data from Keen (1978). [c] Data from Watson and Brooks (1984).

scraped off and eluted with 1 mL of hexane/2-propanol (3:1 v/v). Capsidiol was the main compound in this band as assayed by GLC.

A 200-μL aliquot from the TLC preparation was evaporated to dryness, and then trimethylsilyl ethers were prepared by treating the dried sample with 500 μL of a solution of 400 μL of *n*-heptane/pyridine (2:1 v/v) and 100 μL of *N,O*-bis(trimethylsilyl)trifluoroacetamide/trimethylchlorosilane (10:1 v/v). This mixture was incubated at 70 °C for 15 min, and 1–2-μL sample was analyzed on a Hewlett-Packard GLC with a Durabond DB-1 column, 30 m × 0.32 mm × 0.25 μm. The oven temperature was 190 °C, and the injector and detector temperatures were 220 °C. Helium was employed as the carrier gas at a linear velocity of 21 cm/s and at a pressure of 9 psi. Internal and external standards consisted of *n*-eicosane and capsidiol, respectively.

RESULTS AND DISCUSSION

Five-day-old soybean seedlings treated with 0.25% (w/v) PL accumulated glyceollin during a 3-day incubation period. Although glyceollin production in our system was approximately 40% of the yields reported in other systems (Tables I and II; Bloch et al., 1984; Keen, 1975, 1978), our procedure is less cumbersome and more reproducible than those used in other studies and could be readily adapted for mass production of glyceollin.

Our novel split pea elicitation procedure gave pisatin yields twice those reported for a pod elicitation procedure (Perrin and Cruickshank, 1965; Sweigard and VanEtten, 1987) (Tables I and II) and was faster than a seedling elicitation procedure that gave comparable yields of pisatin.

The procedure we employed for capsidiol accumulation in pepper gave higher yields and would be more practical for mass production than procedures reported previously (Bloch et al., 1984; Watson and Brooks, 1984). Chili peppers also were elicited successfully by PL or yeast extract according to our method (data not included). Young or senescent fruits of sweet pepper, however, did not accumulate significant amounts of capsidiol.

In conclusion, Polytran L, a commercially available fungal glucan, can be used as an elicitor for mass production of phytoalexins in soybean, pea, and pepper. Although glucan elicitors isolated from cell walls of the fungus *Phytophthora megasperma* f. sp. *glycinea* were shown to elicit phytoalexins in plants other than soybean (Darvill and Albersheim, 1984), arachidonic and eicosapentaenoic acids, which act as elicitors of phytoalexin accumulation in potato, do not elicit phytoalexins in many other plant species (Bloch et al., 1984). The spectrum of crop species in which Polytran L elicits phytoalexin accumulation remains to be determined, but our results indicate that Polytran L can elicit phytoalexin accumulation in crops as diverse as legumes and solanaceous plants.

Size separation of commercial Polytran L, using membrane filters, shows that fractions between 3 and 1 kDa have glyceollin elicitation activity, as determined by a soybean cotyledon bioassay (Bhandal and Paxton, unpublished results). Polytran L is not a pure preparation, and the precise structure of the elicitor is not known. Nevertheless, PL can be used as an elicitor and has advantages over the widely used heavy metal salt elicitors because the plant material can be safely used and disposed of after phytoalexin extraction.

ACKNOWLEDGMENT

We thank Dr. John Jerrell, Mass Spectroscopy Laboratory, Department of Food Sciences, for help in GLC of capsidiol. This research was supported in part with funds from the Biological Research and Development Corp. and the Illinois Agricultural Experiment Station.

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Received for review June 10, 1991. Accepted September 17, 1991.

Registry No. Polytran L, 102511-15-9; glyceollin, 66241-09-6; pisatin, 469-01-2; capsidiol, 37208-05-2.